

-continued

Example	EZH2 IC ₅₀ (nM)
27	316
28	631
29	1,259
30	16
31	13
32	50
33	40
34	63
35	40
36	16
37	50
38	100
39	1,259
40	32
41	40
42	20
43	63
51	200
52	631
54	3,981

Assay Protocol 2

[0884] Compounds contained herein were evaluated for their ability to inhibit the methyltransferase activity of EZH2 within the PRC2 complex. Human PRC2 complex was prepared by co-expressing each of the 5 member proteins (FLAG-EZH2, EED, SUZ12, RbAp48, AEBP2) in Sf9 cells followed by co-purification. Enzyme activity was measured in a scintillation proximity assay (SPA) where a tritiated methyl group is transferred from 3H-SAM to a lysine residue on a biotinylated, unmethylated peptide substrate derived from histone H3. The peptides were captured on streptavidin-coated SPA beads and the resulting signal was read on a ViewLux plate reader.

Part A. Compound Preparation

- [0885] 4. Prepare 10 mM stock of compounds from solid in 100% DMSO.
- [0886] 5. Set up an 11-point serial dilution (1:4 dilution, top concentration 10 mM) in 100% DMSO for each test compound in a 384 well plate leaving columns 6 and 18 for DMSO controls.
- [0887] 6. Dispense 10 nL of compound from the dilution plate into reaction plates (Corning, 384-well polystyrene NBS, Cat#3673).

Part B. Reagent Preparation

- [0888] Prepare the following solutions:
- [0889] 7. 1× Base Buffer, 50 mM Tris-HCl, pH 8, 2 mM MgCl₂: Per 1 L of base buffer, combine 1 M Tris-HCl, pH 8 (50 mL), 1 M MgCl₂ (2 mL), and distilled water (948 mL).
- [0890] 8. 1× Assay Buffer: Per 10 mL of 1× Assay Buffer, combine 1× Base Buffer (9.96 mL), 1 M DTT (40 uL), and 10% Tween-20 (1 uL) to provide a final concentration of 50 mM Tris-HCl, pH 8, 2 mM MgCl₂, 4 mM DTT, 0.001% Tween-20.
- [0891] 9. 2× Enzyme Solution: Per 10 mL of 2× Enzyme Solution, combine 1× Assay Buffer (9.99 mL) and 3.24 uM EZH2 5 member complex (6.17 uL) to provide a final enzyme concentration of 1 nM.
- [0892] 10. SPA Bead Solution: Per 1 mL of SPA Bead Solution, combine Streptavidin coated SPA beads

(PerkinElmer, Cat# RPNQ0261, 40 mg) and 1× Assay Buffer (1 mL) to provide a working concentration of 40 mg/mL.

- [0893] 11. 2× Substrate Solution: Per 10 mL of 2× Substrate Solution, combine 40 mg/mL SPA Bead Solution (375 uL), 1 mM biotinylated histone H3K27 peptide (200 uL), 12.5 uM 3H-SAM (240 uL; 1 mCi/mL), 1 mM cold SAM (57 uL), and 1× Assay Buffer (9.13 mL) to provide a final concentration of 0.75 mg/mL SPA Bead Solution, 10 uM biotinylated histone H3K27 peptide, 0.15 uM 3H-SAM (~12 uCi/mL 3H-SAM), and 2.85 uM cold SAM.
- [0894] 12. 2.67× Quench Solution: Per 10 mL of 2.67× Quench Solution, combine 1× Assay Buffer (9.73 mL) and 10 mM cold SAM (267 uL) to provide a final concentration of 100 uM cold SAM.

Part C. Assay Reaction in 384-Well Grenier Bio-One Plates

Compound Addition

- [0895] 3. Stamp 10 nL/well of 1000× Compound to test wells (as noted above).
- [0896] 4. Stamp 10 nL/well of 100% DMSO to columns 6 & 18 (high and low controls, respectively).

Assay

- [0897] 10. Dispense 5 uL/well of 1× Assay Buffer to column 18 (low control reactions).
- [0898] 11. Dispense 5 uL/well of 2× Substrate Solution to columns 1-24 (note: substrate solution should be mixed to ensure homogeneous bead suspension before dispensing into matrix reservoir).
- [0899] 12. Dispense 5 uL/well of 2× Enzyme Solution to columns 1-17, 19-24.
- [0900] 13. Incubate the reaction for 60 min at room temperature.

Quench

- [0901] 5. Dispense 6 uL/well of the 2.67× Quench Solution to columns 1-24.
- [0902] 6. Seal assay plates and spin for ~1 min at 500 rpm.
- [0903] 7. Dark adapt plates in the ViewLux instrument for 15-60 min.

Read Plates

- [0904] 2. Read the assay plates on the ViewLux Plate Reader utilizing the 613 nm emission filter or clear filter (300 s exposure).

Reagent addition can be done manually or with automated liquid handler.

Results

[0905] Percent inhibition was calculated relative to the DMSO control for each compound concentration and the resulting values were fit using standard IC₅₀ fitting parameters within the ABASE data fitting software package.

[0906] Several of the exemplified compounds were generally tested according to the above or an analogous assay and were found to be inhibitors of EZH2. Specific biological activities tested according to such assays are listed in the